PHYLOGENY AND BIOCHEMISTRY OF THE GENUS *HANSENULA*[1]

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I. PHYLOGENY

A. Introduction

The genus *Hansenula* may be defined as yeasts which assimilate nitrate, reproduce asexually by budding or by budding accompanied by pseudohyphae or true hyphae, and produce one to four ascospores in asci that rupture after the spores are formed.

In 1951, it was reported briefly that species of *Hansenula* isolated from coniferous trees are generally haploid, ferment one or rarely two sugars, and produce colonies that are mucoid (36). Species isolated from deciduous trees produce mainly haploid cells but also some diploid, and colonies range from submucoid to butyrous. All species isolated from trees require vitamins for growth in chemically defined media. Species isolated from soil, fruit juices, or other sources independent of trees are predominantly diploid, usually ferment three to five sugars, generally synthesize all the vitamins needed, and often produce mat colonies. Such yeasts apparently represent an evolutionary line in which an increasing ratio of diploid to haploid cells, greater fermentative powers, an increasing ability to synthesize vitamins, and a change from mucoid to glistening butyrous and finally to mat dry colonies indicate a sequence of evolutionary development within the genus. There are, in addition, phylogenetic lines of species limited in habitat to coniferous trees that slowly progressed toward the diploid state and gradually lost the ability to produce a gaseous fermentation. Here is evidence of a tendency to develop toward complete dependence upon trees with a concomitant loss of physiological activity, even to the extent of losing the ability to assimilate nitrate—the principal characteristic by which the genus is recognized.

The most primitive species of the genus *Hansenula* are isolated from present day conifers. If one assumes that the conifers, the bark beetles, and the yeasts evolved together, then it follows that the most primitive yeasts of this genus

[1] This review is based on a paper, entitled "Sucesos sexuales y bioquímicos que caracterizan el desarrollo filogenético del género *Hansenula* [Sexual and Biochemical Changes Revealing the Evolution of the Genus *Hansenula*] by L. J. Wickerham, presented as part of a symposium on the physiology of fungi at the III Congreso Nacional de Microbiologia, Asociacion Mexicana de Microbiologia, Mexico City, D.F., October 12 to 16, 1960.

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evolved some time after modern conifers appeared on earth. This period, so far as the over-all evolution of living forms is concerned, is fairly recent in geological epochs, perhaps within the past 200 million years. No inference is intended, however, that all genera of fermentative yeasts appeared recently on earth. We believe that the rapidity with which a genus progresses toward the diploid state is a good measure of the length of its existence. The more rapidly it progresses, the more recent its origin. Based on this assumption, *Hansenula* is relatively young. In contrast, *Debaryomyces* is old because it consists of species almost all of which are exclusively haploid. The state of the nucleus seems to be largely fixed at the haploid level, even in one branch where species show gaseous fermentation of an increasing number of sugars. This aspect prompted van der Walt (35) to note that among the various sexual or biochemical characteristics which may normally be expected to show evolutionary development among the fermentative yeasts, one may remain nearly constant while another progresses.

There is evidence in *Hansenula* that the homothallic species evolved separately from the heterothallic species. For example, the five most recently evolved species of the homothallic line evolving to independence from trees produce Saturn-shaped spores (Fig. 1, *Hansenula californica* through *Hansenula beijerinckii*, inclusively); yet comparable heterothallic species have exclusively hat-shaped spores. If homothallic species gave rise to both homothallic and heterothallic species, one might expect to find Saturn-spored species in the heterothallic line. Similarly, none of the predominantly diploid species of the homothallic line grow in the medium used to test tolerance for osmotic pressure (10% of sodium chloride and 5% of glucose in a chemically defined medium); whereas all species of the comparable heterothallic line grow, and two out of the three grow exceptionally well (Table 1). Consistent difference in shape of spore and the less consistent degree of tolerance of osmotic pressure suggest that the homothallic and heterothallic lines separated before the advent of the most primitive species known in the genus and have remained separate ever since.

The genus at present consists of five phylogenetic branches. A homothallic (line 1, Fig. 1) and a heterothallic (line 2) chain of species evolved from coniferous through deciduous trees and, finally, into habitats where microorganisms are free living and highly competitive. A homothallic (line 3) and a heterothallic (line 4) chain

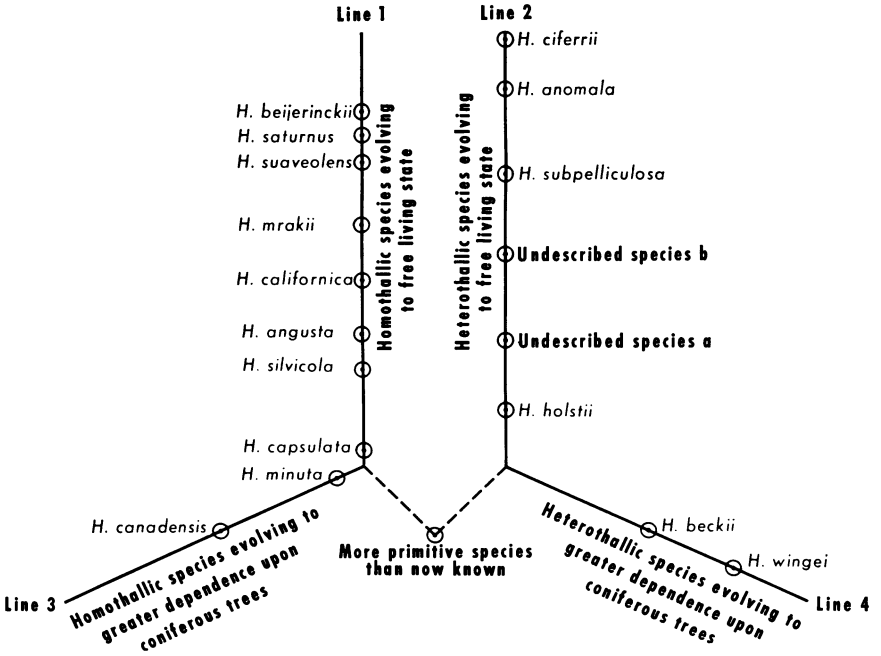

FIG. 1. *Presumed phylogenetic relationships within the genus Hansenula*

evolved toward complete dependence upon coniferous trees. A fifth chain of species, which contains a few species apparently associated primarily with the animal body, need be only mentioned. Lines 1 and 2 contain most of the species already described. Two studied but not published are included as undescribed species *a* and *b* in line 2 to make the heterothallic line more nearly equal in number to the comparable homothallic line.

B. Ploidy in Homothallic Species

Sporulation in *Hansenula capsulata* is commonly consummated as follows. The haploid nucleus of an adult cell divides mitotically. One of the nuclei passes into a bud and back again to combine with the one remaining in the adult cell. The bud dies. The nucleus is momentarily diploid, then it undergoes reduction division to form two haploid nuclei, each of which produces a hat-shaped cytoplasmic structure around itself to become an ascospore. The ascus ruptures; the liberated spores increase in size and then give rise to haploid buds, which continue the haploid vegetative phase of the yeast. Conjugation rarely occurs between two independent cells whose nuclei fuse, then the diploid nucleus undergoes reduction division. The diploid state is thus limited to a very short period in the life of the yeast. Such yeasts are commonly designated as haploid species. It is probable that a pair of conjugated cells, or zygote, in extremely rare instances produces a diploid bud (i.e., a diploid vegetative cell). Diploid vegetative cells probably occur but are so infrequent that *H. capsulata* has not yet been isolated from nature in the diploid state. If a diploid culture were isolated, it would sporulate without conjugation, and each ascus would be a single cell *without* a dead bud. Conjugated asci are characteristic of haploid species.

Sporulation in *Hansenula silvicola* is similar to that in *H. capsulata*, except that some 4% of the asci are not conjugated. The latter arose from diploid cells whose more or less distant forebears were buds on a zygote that did not immediately proceed to sporulate but, instead, produced diploid buds. Such diploid cells can be isolated and can produce pure diploid cultures so long as sporulation is prevented.

In *Hansenula angusta* almost all cells are haploid as they exist in frass under the bark of deciduous trees or in acorns serving as homes for larval stages of insects. There are some diploid cells, too, and at this level of phylogenetic development diploid colonies, if well isolated, grow slightly larger and sporulate sooner than do haploid colonies. The ascospores impart a red color to the colonies; consequently the diploids when about 6 days old are distinguished from the haploids by their deeper red color. Once a diploid colony sporulates, however, it becomes a mixture of haploid and diploid cells because ascospores are haploid, except in the rather rare cases of sporulated yeasts having three or four sets of chromosomes. Haploid colonies show mainly conjugated asci, but some have unconjugated asci since the species attempts to exist partly in the diploid state. However, cultures kept indefinitely on rich laboratory media may become exclusively diploid because sporulation is generally suppressed and because diploid cells eventually outgrow haploid cells even at a low level of development.

Teunisson, Hall, and Wickerham (32) have suggested *H. angusta*, because of its pigmentation, as particularly good for study of the coexistence of haploid and diploid cells. *H. californica* also exists in nature predominantly as haploid cells, but long cultivation on laboratory media often causes them to be replaced entirely by the slightly faster growing diploids.

Hansenula mrakii is at an evolutionary level which causes it to exist in nature at about 90% diploid cells. A few serial transfers made at approximately weekly intervals on sporulation medium will show a majority of conjugated asci, indicating that the ascospores commonly give rise to haploid vegetative cells which reproduce considerably before diploidizing. Serial transfers on cultivation medium, however, may give 90% or more unconjugated asci, indicating that this medium favors diploid cells. Since cultivation medium supports more growth than does sporulation medium, the diploids have a greater opportunity to outgrow the haploids. It is apparent that *H. mrakii* has a greater tendency to diploidize in nature and in the laboratory than do the preceding species. Laboratory cultivation of *H. mrakii* may lead rapidly to pure diploid cultures because of the greater rate of growth of the diploid cells at this higher level of evolutionary development.

Hansenula suaveolens, *Hansenula saturnus*, and *H. beijerinckii* in nature and in cultivation are

some 99% diploid, which means that the original isolation colonies are either diploid or almost exclusively diploid in composition.

C. Ploidy in Heterothallic Species

Primitive heterothallic species are often quite refractory to mating for reasons to be presented in a forthcoming monograph on the evolution of sexual processes in yeasts. Some 40 strains of *Hansenula holstii* were mixed together on sporulation slants before two were found that would conjugate to give some 3 to 5% of spores after 3 weeks of incubation. From such a sporulated culture, 10 ascosporic cultures were isolated by a heat treatment that kills the vegetative cells and many, but not all, of the spores (42). One ascosporic isolate, when mated to the parent of the opposite sex, yielded many spores in 8 days. This sporulated culture was also heat treated, and all the resulting ascosporic isolates tested sporulated abundantly when mated to siblings of opposite sex. In 1956, Dr. J. Lodder, as recipient of a grant from the American Association of University Women, spent some months at the Northern Regional Research Laboratory studying our concepts of phylogeny. While studying the sexuality of *H. holstii*, she obtained a vegetative diploid culture that sporulates abundantly. The cells of the diploid, but not its colonies, are considerably larger than those of the haploid. In 1960, the sexuality of *H. holstii* was reported in considerable detail (41).

The undescribed species *a* in Fig. 1 was almost as refractory as *H. holstii* in its original sexual reactions, although in a different way, and mixtures of opposite mating types gave no spores or perhaps one per billion vegetative cells. However, by inbreeding as practiced with *H. holstii*, more fruitful mating types were produced, and an abundantly sporulating diploid culture has now been obtained.

Undescribed species *b* (line 2) is a free-living yeast. Nearly all strains are reactive when mixed with an active tester of the opposite sex. The cells conjugate abundantly, and while many of the newly formed zygotes proceed immediately to sporulate, others produce diploid vegetative buds. Diploid vegetative cells may be readily isolated as diploid colonies on plates streaked from the sporulation mixture. In nature, however, the species evidently maintains itself exclusively in the haploid form. Heterothallic species, poised near the evolutionary level at which the shift from predominantly haploid cells to diploid occurs, may be influenced considerably by the medium to take one form or the other. Rapid serial growth of a mixture of the sexes on cultivation medium favors the haploid form of undescribed species *b*. Rapid growth on sporulation medium favors the diploid form because conjugation is stimulated.

Hansenula subpelliculosa exists both in nature and in the laboratory almost entirely as diploid cells. Immediately upon germination of ascospores, the opposite sexes begin to conjugate and the zygotes to produce vegetative diploid cells. After two or three daily transfers, practically all the haploid cells are eliminated from the culture due to the more rapid growth of the bisexual cells. Since the species is heterothallic and ordinarily can increase its ploidy only by mating with the opposite sex, haploid cells in nature can be isolated and will continue as pure haploid cultures. Colonies of the diploids of *H. subpelliculosa*, *Hansenula anomala*, and *Hansenula ciferrii* are much larger than haploid colonies of the same age. As previously intimated, the higher the evolutionary development of the species, the greater the difference in rate of growth of the haploid and diploid forms.

H. anomala, to a slight extent, and *H. ciferrii*, to a greater extent, evidently exist in old laboratory cultures as polyploids. They are not observed to do so in detectable numbers in nature. It seems plausible that the continuation of lines 1 and 2 of Fig. 1 during forthcoming epochs shall result in species showing increasingly substantial ratios of polyploid cells in nature.

Homothallic line 3 contains a few undescribed and described haploid and diploid species. Ascosporic isolates of the latter often show a weak tendency to diploidize, and they may remain haploid for several transfers. Conversely, diploid species in homothallic line 1, such as *H. saturnus*, produce ascospores that begin to diploidize soon after germination. Heterothallic line 4 contains *Hansenula wingei* (38), which exists in nature both as diploid and haploid cells. Some diploid cultures yield opposite mating types that do not mate when mixed but to a limited extent do so some months after ascosporic cultures have been isolated. Other diploids isolated from the same geographical area yield mating types that clump together when cells of the opposite sexes come

into contact. This process is called sexual agglutination. About 1 hr after clumping, the cells conjugate in pairs and produce diploid buds. Thus sexual agglutination overcomes a weakened tendency to diploidize. We shall consider this process again in Section II of this review.

D. Number of Sugars Fermented and Intensity of Fermentation

Table 1 reveals that almost all haploid species ferment only one or two sugars and that the diploid species ferment three to five sugars, but a stepwise increase from species to species does not occur. Intensity of fermentation is shown by the average number of days elapsing between inoculation of the glucose fermentation tube and the reduction of gas volume in the insert. A general increase in rapidity of fermentation occurs with ascending evolutionary position in the fermentative lines. *H. holstii* is more strongly fermentative than its position would suggest it might be.

TABLE 1. *Characteristics on which phylogeny of the genus Hansenula is based**

Hansenula species	Habitat	Ploidy	Fermentation					Length of fermentation	Production of esters	Vitamins required	Colony type	Growth at moderate osmotic pressure
			G	Gal	M	S	R 1/3					
								days				
Homothallic Line 1, Fig. 1												
H. capsulata	C	H	+	−	−	−	−	12	−	+	Mu, b	Sc
H. silvicola	D	H, d	+	+	−	−	−	13	−	+	mu, B	−
H. angusta	D	H, d	+	−	−	−	−	10	−	+	G, B	Mod
H. californica	F	H, d	+	−	−	−	−	9	+	+	G, B	−
H. mrakii	F	h, D	+	−	−	−	−	5	+	−	M, g, B	−
H. suaveolens	F	h, D	+	−	−	+	+	6	+	−	M, g, B	−
H. saturnus	F	h, D	+	−	−	+	+	5	+	−	M, g, B	−
H. beijerinckii	F	h, D	+	−	−	+	+	6	+	−	M, B	−
Homothallic Line 3												
H. minuta	?	H	V	−	−	−	−	17	−	+	mu, b	−
H. canadensis	C	D	−	−	−	−	−	—	+	+	G, B	−
Heterothallic Line 2												
H. holstii	C, d	H	+	V	−	−	−	8	−	+	Mu, b	Mod
Undescribed species *a*	C	H	L	L	−	−	−	14	−	+	G, B	−
Undescribed species *b*	F	H	+	−	L	+	+	8	+	+	G, B	Mod
H. subpelliculosa	F	h, D	+	−	V	+	+	8	+	+	G, B	Ab
H. anomala	F	h, D	+	V	V	+	+	5	+	−	G, M, B	Ab
H. ciferrii	F	D	+	+	V	+	+	7	+	−	g, M, B	Sc
Heterothallic Line 4												
H. beckii	C	?	−	−	−	−	−	−	−	+	g, M	−
H. wingei	C	h, D	−	−	−	−	−	−	+	+	G, B	−

* Capital letters denote strong tendencies or reactions, small letters denote weak tendencies or reactions:

Habitat: C, coniferous trees; D, deciduous trees; F, free living in soil, water, or exudates of plants.

Ploidy: H, haploid; D, diploid.

Fermentation: + indicates evolution of gas; − indicates no evolution of gas; G, glucose; Gal, galactose; M, maltose; S, sucrose; R 1/3, raffinose 1/3.

Length of fermentation: number of days from time of inoculation until evolution of gas has stopped.

Production of esters: + indicates odor of ethyl acetate; − indicates weak sweet odor not characteristic of ethyl acetate, or no odor of esters at all.

Vitamin requirement: + indicates growth in chemically defined medium without addition of vitamins; − indicates no growth in chemically defined medium without addition of vitamins.

Colony type: Mu, mucoid; B, butyrous; G, glistening; M, mat.

Growth at moderate osmotic pressure: Ab, abundant; Mod, moderate; Sc, scant; −, none.

E. Ester Production

The ability to produce ethyl acetate becomes and remains positive at a certain evolutionary level in both lines 1 and 2, Fig. 1.

F. Vitamin Requirements

The ability to synthesize all required vitamins was achieved at a definite point in lines 1 and 2, and all succeeding species possess this ability. All species in lines 3 and 4 evidently retain a marked need for vitamins. In 1953, Furutani, Betz, and Hedrick (12) determined which vitamins are required for growth and which ones are not absolutely required but do hasten the growth of the yeast in a vitamin-free basal medium. Their work was done before some of the species given in Fig. 1 were discovered, yet the genus was rather well represented. According to them, *Hansenula beckii* and *Hansenula canadensis* in lines 4 and 3 required biotin, thiamine, and pyridoxine. *Hansenula minuta* required biotin and thiamine, but evidently a third vitamin may be required since growth of the culture was poor even with biotin and thiamine added to the basal medium. *H. capsulata* and *H. silvicola* required biotin, thiamine, and pyridoxine for good growth, and *H. angusta* and *H. californica* required only biotin. Species higher than *H. californica* in line 1 required no vitamins. In line 2, *H. subpelliculosa* required thiamine, and *H. anomala* and *H. ciferrii* required no vitamins. So far as the species of *Hansenula* were covered by this study, the species developing toward greater dependence upon trees (lines 3 and 4) have the maximal need for vitamins; those developing toward a free-living state became less dependent and require less vitamins; and the most highly evolved species of lines 1 and 2 produce all the vitamins they need.

G. Characteristics of the Colony Surface

Next in importance to changes in ploidy are believed to be changes in appearance of colonies. Colony changes reflect differences in the basic needs of the species at various stages of evolution and in the types of biochemicals that satisfy these needs. The first need, partially at least, is for a sticky substance to hold primitive yeast cells onto the bodies of bark beetles and to prevent death by dehydration during flight of the beetle from one tree to another. The sticky, hydrophilic capsular material produced by the yeasts imparts a highly glistening, mucoid appearance to the colony, and the colonies tend to flow if the plate is tipped at a steep angle. Occasionally *H. capsulata* and more commonly *H. holstii* produce butyrous colonies in addition to the mucoid colonies. *H. silvicola* commonly produces highly glistening, butyrous colonies on cultivation medium but produces submucoid colonies on sporulation (malt extract) medium. Above *H. silvicola* and *H. holstii*, through *H. californica* and *H. subpelliculosa*, respectively, the species produce colonies that are glistening and butyrous. The intensity of fermentation increases, and maximal production and retention of alcohol for the genus are achieved by *H. subpelliculosa*. Thus, in liquid media, the accumulation of alcohol primarily, as well as organic acids and esters, causes toxicity and constitutes poorly available carbon for further growth. Species at these levels of development, unequipped to produce strong pellicles at the interface of liquid and air, are thus unable to use economically such intermediary metabolites for growth and energy.

All species higher than *H. californica* and *H. subpelliculosa* develop cell walls to which are anchored hydrophobic molecules that aid some of the cells to float. The mechanisms and chemicals involved are evidently numerous. In some genera, as in *Saccharomyces*, particularly those strains used to make sherry wine, the yeast produces alcohol first, then the cells rise to the surface. In the strongly oxidative, diploid species of *Hansenula*, some cells of the inoculum go immediately to the top and grow there, while more go the the bottom; the bottom cells produce fermentation products and the top cells oxidize them.

The change from hydrophilic to hydrophobic cells is reflected culturally by stronger, thicker, drier pellicles on liquid media, and by a change from glistening to mat colonies and finally to extremely hydrophobic cells that are powdery, white, and dry. The surface cells of mat colonies may be floated free by gently pouring water into the plate. This ability to float suggests that oxygen enhances whatever chemical transformations make the surface cells more strongly hydrophobic. In some strains of diploid species of lines 1 and 2, mat and glistening portions may be observed in the same colony, or some colonies may glisten

while others are mat. This flexible situation may be accentuated in either direction depending upon whether the culture comes into contact with aerobic or anaerobic conditions. Diploid cultures of *H. anomala* are excellent for changing, neither too readily nor too slowly, from pure glistening cultures to pure mat cultures. The species is isolated from nature in both forms, and freshly isolated diploids usually produce ascosporic isolates that range from glistening to mat. Mat forms of *H. ciferrii* and *H. anomala* are more likely to become polyploid than are glistening bisexuals. Polyploids often produce larger colonies and many bisexual ascospores. Some of what is known that relates specifically to the biochemistry of the cell surface of *Hansenula* will be presented in Section II of this review.

Hedrick (14) studied the relative hydrophilic and hydrophobic characteristics of cells of the genus *Hansenula* as determined by the amount of salt required to cause cells to clump. The strength of the reaction is reflected in the viscosity of the sedimented cells and in the patterns formed by the cells on the bottoms of tubes containing the various dilutions of sodium chloride. The saline concentrations used varied from 16 to 4,000 μg per ml.

The five species that are the most hydrophilic live in association with trees. They are *H. holstii*, *H. canadensis*, *H. wingei*, *H. silvicola*, and *H. capsulata*. All produce mucoid or glistening butyrous colonies. The most hydrophilic of all the strains studied was *H. holstii* NRRL Y-2154. The opposite mating type, Y-2155, was less hydrophilic. Y-2155 readily produces nonmucoid colonies that are considerably less hydrophilic, and the nonmucoid form may have been studied.

The next six species are less hydrophilic than the first five. They are *H. subpelliculosa*, *H. californica*, *Hansenula jadinii*, *H. saturnus*, *H. minuta*, and *H. angusta*. The first four are free living, *H. angusta* lives in association with deciduous trees, and the habitat of *H. minuta* is not known with certainty. All produce glistening butyrous colonies although certain strains of *H. saturnus* produce some mat colonies or partially mat colonies. The remaining five species have the most hydrophobic cells. They are *Hansenula schneggii*, *H. ciferrii*, *H. mrakii*, *H. suaveolens*, and *H. anomala*, all of which produce predominantly or exclusively mat colonies. Hedrick's order of increasing hydrophobicity generally correlates with the types of colonies which the species produce and, therefore, with phylogeny, since colony characteristics are among the most important on which phylogeny of *Hansenula* is based.

H. Antigenic Analysis

Tsuchiya et al. (33) have published several papers on the antigenic analysis of yeasts in various genera. In 1958, the antigens were listed by number for some of the more common species of *Hansenula* (33). All were thermostable and are as follows: *H. californica*, 1, 2, 14, 15, 16, 17, 20, 21, 22; *H. saturnus*, *H. suaveolens*, and *H. mrakii*, 1, 2, 14, 15, 16, 17, 20, 21. *H. anomala*, *Candida pelliculosa*, *H. schneggii*, *H. subpelliculosa*, and *H. silvicola* all possessed identical antigens, 1, 2, 14, 15, 16, and 20. *C. pelliculosa* is the name sometimes used for nonsporogenous strains of *H. anomala*, most of which mate with an appropriate tester. *H. schneggii* is an extremely mat form of *H. anomala*. At present Tsuchiya's serological procedure cannot be used to separate all species of *Hansenula*. We believe that judicious application of his procedure to phylogenetic problems in yeasts, particularly in the troublesome areas of *Debaryomyces*, *Pichia*, and *Endomycopsis*, would yield valuable returns. It could also be useful in substantiating or limiting the possible relationships of newly discovered genera to other existing genera as suggested by the discoverers. Specifically, van der Walt (34) has noted the possible relationship of his new genus *Kluyveromyces* with the group of yeasts which Wickerham and Burton (43, 44) intend to describe as the new genus *Dekkeromyces*. Similarly, the new genus *Wickerhamia* is believed by Soneda (28) to be related, possibly, to *Spermophthora*, *Ashbya*, and *Eremothecium*.

I. Genera Closely Related to Hansenula

Two genera that share a common origin with *Hansenula* will be noted briefly here.

Pachysolen was erected by Boidin and Adzet (2) for two species which produce asci that consist of a small chamber containing hemispheroidal ascospores set in the end of a long cylindroidal cell containing much refractile material. So far as we know, *Pachysolen* and *Hansenula* have a similar physiology.

A large nitrate-negative genus evidently arose from primitive species of *Hansenula*. The ratio

of tree-inhabiting species is higher in it than in *Hansenula*, a smaller percentage of the species is strongly fermentative, and very few produce all the vitamins they require. This group will probably contain the type species of *Pichia* as the oldest type species included within it. Phaff (20) has offered a loose definition of the genus, but as yet he has not indicated the extent of the known species that should be placed within it.

This phylogenetic analysis of *Hansenula*, a currently average-size genus of yeasts, suggests that certain trends have occurred in specific directions; the characteristics shown by any species indicate where in the evolutionary sequence it was born. The major characteristics depicting the phylogeny of the genus are habitat; ploidy; mucoid, glistening, or mat appearance of colonies; number of sugars fermented and the intensity of fermentation; vitamin requirements; and ester production. As more species are discovered, and as more biochemical studies are made, the strength of the theory and the precision with which the species may be placed in evolutionary order should increase. We have discovered additional species of tree-inhabiting *Hansenula* and shall describe them soon. All fit into our postulated scheme and add support for it.

II. Biochemistry

A. Introduction

This section deals largely with types of molecules produced on the surface of cells of the most primitive species of *Hansenula* and also of the most highly evolved. Species of other genera that are either related phylogenetically or that possess a common biochemical mechanism are also included. Before discussing specific compounds, we shall present our views on the production of large, extracellular molecules by yeasts in general.

We believe that certain large surface molecules, such as starch, phosphomannan, invertase, and sphingolipids, are produced by the cell either internally or externally and then are anchored to particular molecules on the surface of the cell to the extent that anchoring sites are available. Beyond this capacity, the large molecules are liberated into the surrounding medium. If seeing is believing, the following observations are in order. Yeasts and yeastlike organisms that produce scant amounts of starch (37) often produce all of it on the cell walls where it is stainable with iodine; the supernatant medium does not yield the blue color. Instead, yeasts that produce starch abundantly excrete so much into the medium that iodine renders the supernatant blue-black. Phosphomannans are largely excreted, yet microscopical observation shows a wide zone of phosphomannan forming a capsule around the cells. Invertase is excreted in small amounts, if at all, by most yeasts, yet some excrete up to 25% or more of the total; actually the bulk of the invertase remains inside or attached to the cell wall. Under "E. Invertase" of this section we shall delve into the production of invertase within the cell and follow its possible route to excretion into the surrounding medium.

B. Phosphomannans

Primitive species of *Hansenula* and closely related genera produce extracellular mannans that contain characteristic numbers of phosphate groups (1, 26, 45). The phosphomannans, as this new class of microbial polymer has been designated (15), are highly soluble in water and form a sticky layer on the surface of yeasts. Apparently they promote dispersal of yeasts by bark beetles.

All phosphomannans are depolymerized by acid hydrolysis to D-mannose and D-mannose-6-phosphate (25). The molar ratio of mannose to phosphate (Table 2) depends largely upon the species producing the phosphomannan and appears to be independent of the composition of the medium (1). The most highly phosphorylated mannans are produced by the most primitive species; the less highly phosphorylated, by the less primitive (26). It is assumed that the most highly evolved species of *Hansenula* produce mannans that are not phosphorylated at all and that are not liberated appreciably into the culture medium.

According to the criteria by which evolutionary progress is determined for species of *Hansenula*, *H. capsulata* is the most primitive of the genus. Its phosphomannan, as represented by the formula in Fig. 2 (M. E. Slodki, *private communication*), contains the most phosphate. Some 20% of the β-1,2-linked backbone unit contain a single α-linked mannose side chain unit not shown in the structural formula. Strains of *H. holstii* produce phosphomannans containing half as much phosphate. The hypothetical formula of Jeanes and Watson (16) for Y-2448 phosphomannan, shown in Fig. 3, gives the structural types

TABLE 2. *Correlation of phylogenetic position of yeasts with properties of the phosphomannans that they produce, particularly the mannose to phosphate ratio (26)**

Species	Ability to assimilate nitrate	Habitat	Ploidy	Sugars fermented	Ability to produce esters	No. of strains studied	M:P ratio	$[\alpha]_D^{25}$	D. P.	Viscosity
Hansenula, Lines 1 and 2, Fig. 1										
H. capsulata	+	C	H	G	−	4	2.4–2.6	−2° to +21°	81–244	29–33
H. holstii	+	C, d	H	G, gal	−	9	4.4–5.7	+74° to +106°	274–796	17–46
Hansenula sp. *c*, undescribed	+	C, D	H	G, m	+	3	3.6–3.8	+77° to +100°	115–121	20
Pachysolen										
P. tannophilus	+	D	h, D	G	+	1	4.2	+46°	256	
Hansenula, Line 3										
H. minuta	+	?	H	g-0	−	2	9.8–27.5	+88° to +89°	20–144	11
Hansenula sp. *d*, undescribed	+	C	h, D	0	−	1	11.9	+100°	155	
Pichia, Line 2										
Pichia sp. *a*, undescribed	−	D	H	G, s	+	1	7.2	+101°	17	13
Pichia, Line 3										
Torulopsis pinus	−	C	H	G-g	−	2	8.4–8.5	+68° to 102°	28–51	31–71
Saccharomyces pini	−	C	H, d	g-0	−	6	9.2–13.0	+91° to 102°	24–51	11–83

* Capital letters denote strong tendencies or reactions, small letters denote weak tendencies or reactions:

Habitat: C, coniferous trees; D, deciduous trees; C,d, principal habitat in coniferous trees but found to a less extent in deciduous trees.

Ploidy: H, haploid; D, diploid; h,D, predominantly diploid but haploid cells are frequently isolated.

Sugars fermented: 0, no gaseous fermentation; g, glucose weakly fermented; G, glucose moderately to strongly fermented; gal, galactose weakly fermented; m, maltose weakly fermented; s, sucrose weakly fermented.

M:P ratio: Mannose to phosphorus molar ratio.

$[\alpha]_D^{25}$: Specific optical rotation (1.0% in 0.1 M KCl (15)).

D. P.: Apparent degree of polymerization (alkaline Cu reduction).

Viscosity: In centistokes (0.5% solutions).

and proportions of constituent mannose units, as well as the position of attachment of the orthophosphate groups. Some details of sequence and the absence of branching have not yet been established. All phosphomannans, regardless of degree of phosphorylation, are phosphodiesters in which one of the two secondary phosphoryls is linked as mannose-6-phosphate and the other as α-hemiacetal phosphate (25).

H. holstii is slightly, but definitely, more highly evolved than *H. capsulata*. The latter species is found only in association with the genus *Pinus*; it ferments only glucose, at a moderate rate. *H. holstii* is not limited exclusively to coniferous trees but is also found in gums of *Prunus*; it ferments glucose strongly, but occasional strains also ferment galactose weakly.

Species of *Hansenula* occurring in lines 3 and 4 of Fig. 1 are limited to associations with coniferous trees. Gradually certain physiological abilities were lost as the species evolved successively. These species produced phosphomannans containing fewer phosphate groups than the species in lines 1 and 2. *H. minuta* strain NRRL Y-411 is an outstanding example with a polymer having a ratio of 27 mannose units to 1 phosphate group (Table 2).

Other important phylogenetic relationships are associated with (i) ratio of mannose to phosphorus, (ii) apparent degree of polymerization,

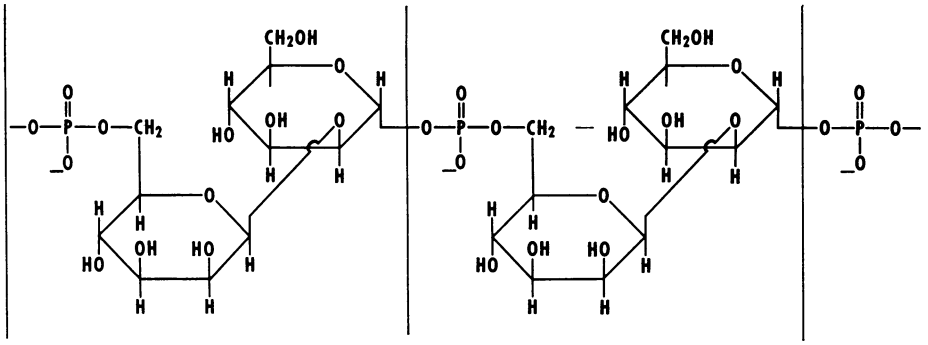

FIG. 2. *Phosphomannan produced by Hansenula capsulata Y-1842*

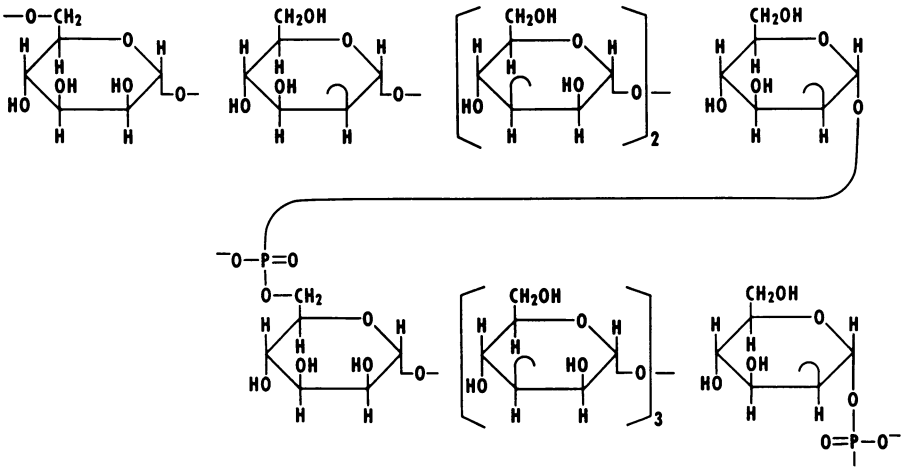

FIG. 3. *Phosphomannan produced by Hansenula holstii Y-2448*

and (iii) production of these newly discovered phosphomannans by three related groups of yeasts. The first relationship involves a large genus of yeasts that arose from primitive species of *Hansenula*. The known species have been assigned to various genera but eventually will be included in the genus *Pichia* when it has been amended to set the limits of the genus precisely. Phaff (20) has taken the initial step in this direction.

Species of *Hansenula* assimilate nitrate, but species of *Pichia* do not. Primitive species of *Pichia* arose mainly from primitive species of *Hansenula* of lines 3 and 4 in Fig. 1, and, as could be expected from such forebears, phosphomannans of the nitrate-negative species have a high mannose to phosphate ratio (Table 2). Phosphomannans of the nitrate-negative species have lower apparent degrees of polymerization as determined by alkaline copper reduction. This value is not necessarily a reflection of molecular size (26). The viscosity values provide no useful correlations but are presented for general information.

The second taxonomic affinity strengthened by the discovery of phosphomannans is the relationship of *Pachysolen* to *Hansenula*, a similarity not anticipated by the discoverers of this relatively new genus (2). The phosphomannan produced by *Pachysolen tannophilus* has a mannose to phosphate ratio intermediate between that of *H. capsulata* and *H. holstii*; it is also intermediate with respect to specific optical rotation (Table 2). High positive specific rotation indicates a preponderance of α-glycosidic linkages in the molecule; low rotation indicates a preponderance of β-glycosidic linkages.

The potential industrial uses of phosphoman-

nans are enhanced by the large yields obtained from certain species. Good yields were first obtained by Dr. Robert G. Benedict. Anderson and co-workers (1) and Rogovin, Sohns, and Griffin (23) in laboratory and pilot-plant scales, respectively, obtained 50% of extracellular polymer based on the amount of sugar supplied. This amount is exclusive of that in the capsules of the cells. Aqueous solutions of Y-2448 phosphomannan are highly viscous and thixotropic, and lack toxicity, color, odor, and taste (15). Viscosity is increased by adding suitable concentrations of borax, which causes complexing and crosslinking of the molecules. The presence of potassium chloride further enhances this effect (15). Slodki (24) opened the diester groups by mild autohydrolysis of phosphomannans to obtain polymannosidic phosphomonoesters of chain lengths consistent with the mannose to phosphorus ratios of the intact polymers. Thus, a phosphorylated disaccharide was the predominant molecular species obtained from the phosphomannan produced by *H. capsulata*; cleavage occurs at the places indicated by the vertical lines in the structural formula. A phosphorylated pentasaccharide was the predominant molecular species obtained from the phosphomannan produced by *H. holstii* Y-2448 (24).

It is hoped that the native phosphomannans and their component polymannosidic phosphomonoesters obtained by mild hydrolysis may find uses as thickening, stabilizing, dispersing, and suspending agents.

C. Zymonic Acid

No attempt has yet been made to determine which species of *Hansenula* produce zymonic acid and which do not. Nor do we know whether other species make modifications of the molecular structure produced by *H. subpelliculosa*. Consequently, we do not know whether the acid has phylogenetic significance. Since zymonic acid is produced by yeasts in widely different taxonomic groups, its common occurrence in intermediary pathways of yeasts is suggested, making its common name from the Greek word *zymē* (meaning leaven or yeast) quite appropriate.

Stodola, Shotwell, and Lockwood (30) isolated zymonic acid in the form of its methyl ether, to which an α,β-unsaturated acid structure was tentatively assigned. A closer examination of the problem by Haynes and Plimmer (13) has shown zymonic acid to have the alternate tetronic acid structure shown in Fig. 4. Ascorbic acid, a group of complex pigments found in lichens, and a number of metabolites produced by several species of *Penicillium* are all tetronic acid derivatives.

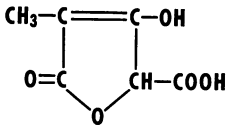

FIG. 4. *Zymonic acid*

D. Sphingolipids

Yeasts bear on their surfaces molecules that are characteristic of their evolutionary state of development. In *Hansenula*, the primitive species have hydrophilic walls by grace of the phosphomannans they produce. The most highly evolved species are both fermentative and oxidative, the latter property being augmented and expressed by an ability to produce pellicles on liquid media. Pellicle formation is associated with, and dependent to a large extent upon, the elaboration of fats inside and outside the cells, thereby lessening specific gravity and increasing hydrophobicity—both factors favoring the production of pellicles. Wickerham and Stodola (47) found that the most highly evolved species of its line, *H. ciferrii*, possesses the unique property of producing crystalline sphingolipids. Stodola and Wickerham (31) reported the most abundant to be tetraacetylphytosphingosine, a new compound.

Mating types in one sex of *H. ciferrii* are particularly prone to produce microcrystals in cultures. The mat (or oxidative) colony forms of this sex produce them more abundantly than the glistening (or fermentative) colony forms. Most of the microcrystals melt at a low temperature, a few do at higher temperatures. The crystalline form sets them apart from other sphingolipids; those commonly occurring in plants and nerve tissue of animals are chemically bound to other types of molecules, and this bonding makes their isolation and purification difficult. Tetraacetylphytosphingosine contains a long hydrocarbon chain to which are attached three *O*-acetyl groups and one *N*-acetyl group (Fig. 5).

Cells of the strongly mat mating types of *H. ciferrii*, when grown in liquid media to which

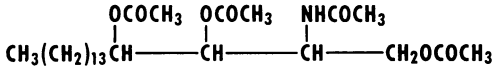

FIG. 5. *Tetraacetylphytosphingosine*

either asbestos or nylon fibers or crystals of acenaphthene have been added, adhere strongly to the fibers and crystals to escape the liquid. Similarly, when grown on slants or in shaken liquid media at 22 to 25 C, the most strongly hydrophobic cells of the culture form in clumps; the clumps in liquid media often contain, in addition to the cells, practically all the crystals of sphingolipids that the culture has produced. The other crystalline sphingolipids which *H. ciferrii* synthesizes will be described in later papers.

E. Invertase

The site of invertase production and the obstacles the enzyme encounters on its exit from the cells of certain strains have received considerable attention during the past few years. Major attention was directed to industrial strains of *Saccharomyces*, although *Hansenula* and related nonascosporogenous yeasts have been important in this field. In 1957, Myrbäck (18) reported that in the baker's yeast he studied, the enzyme in its native state is insoluble and tightly bound to other cell constituents. Burger, Bacon, and Bacon (4) believed that the invertase in another strain of baker's yeast was soluble; after they crushed the live yeast a large proportion of its invertase was liberated in a water-soluble form. On treatment of live cells with crop juice of the edible snail *Helix pomatia*, about 50% of the invertase was liberated, while at least 90% of the cells remained viable. The snail enzyme is known to attack the cell wall of *Saccharomyces cerevisiae*.

The cell wall of the strain of baker's yeast studied by Northcote and Horne (19) consisted of protein, approximately 13% lipid (mainly neutral fat), 8.5%; and at least two polysaccharides, a mannan, 31%; and a glucan, 29%. The mannan, which is associated with the protein, remains intact after removal of the lipid but disappears when mannan and protein are removed chemically. The glucan can be isolated free from other material and still retain the general shape of the whole cell. An excellent electron photograph of the cell wall from which lipids have been extracted shows (Plate I, Figure 5, reference (19)) the two layers remarkably well.

According to the interpretation of Cook (5), the major constituent of the wall is an insoluble glucan matrix (ca. 50%) attached on inner and outer sides by a protein cement (ca. 7%) to both soluble mannan (ca. 20%) and to soluble glucan (ca. 10%).

The action of snail enzyme is limited to the cell wall and is, therefore, commonly used to produce protoplasts; thus the liberation of invertase from live yeasts by snail laminarase indicates its usual imprisonment in yeasts just below the cell wall. This site of invertase presumably agrees with the findings of Preiss (21), who used a procedure involving low voltage electrons to show that the invertase zone of yeast cells has its inner boundary not greater than 1,000 A and its outer boundary at a depth of about 500 A.

Wickerham (40) and Dworschack and Wickerham (6) reported that live cells of certain strains of *Saccharomyces*, while rapidly growing, excrete into the medium vastly more soluble invertase than do the few dead cells present. Consequently, they (7) started a survey of the ability of yeasts in general to secrete invertase. When high yields were encountered in *H. jadinii*, which is a very close relative if not indeed the weakly ascosporogenous form of the feed yeast *Candida utilis*, the survey was directed to industrial strains because of the commercial uses of invertase. Top yields among 10 strains of *C. utilis* were over 800 units of extracellular and 3,400 units of total invertase per milliliter of culture. Yields were well in excess of those from 33 industrial strains of *S. cerevisiae*, *Saccharomyces carlsbergensis*, and *Saccharomyces steineri*. Marked variations occurred among strains of a single species. *Saccharomyces* are used for invertase production, but *C. utilis* has not yet been so employed.

Friis and Ottolenghi (11) reported that the location of invertase within the strain they studied depended upon whether the yeast was grown in glucose or in sucrose medium. Glucose-grown cells had only intracellular enzyme too deep within the cells to be liberated by snail enzyme. When walls of sucrose-grown cells were dissolved away by snail juice, some 74% of the invertase was liberated. Friis and Ottolenghi believe that invertase is produced internally, but

that after sucrose enters the cell, the enzyme moves through the cell membrane, and that further movement is impeded by the cell wall. They thought that the efficiency of the cell wall to retain invertase varied with the strain of yeast, since Wickerham (40) reported that numerous species elaborate extracellular invertase. Adaptations by grace of a sucrose medium are not required by all yeasts as a prerequisite for peripheral or external location of invertase; Dworschack and Wickerham (7) reported that *C. utilis* produces high yields of extracellular invertase when grown in media having glucose, xylose, glycerol, or ethyl alcohol as carbon source.

Since proteins and mannans are believed by Eddy and Rudin (9) to be involved in agglutination, it may be that bonding between invertase and mannan, or other carbohydrates, occurs as invertase passes through the layers of the cell wall. Absorption of enzymes from aqueous solutions by a strain of *S. cerevisiae* was reported by Kursanov and Isaeva (17). The amounts absorbed depend upon the pH of the solution, the type of enzyme, and the age of the cells. Some enzymes were tenaciously held, whereas others, such as invertase, were so loosely combined that they were replaceable by other enzymes. If enzymes from without the cell may be absorbed, it is likely that enzymes from within are likewise absorbed. The nature of the bond between invertase and the cell surface would influence the firmness with which the invertase was held, and variations that might occur within the structure of invertase and mannan molecules could determine the looseness or tenacity of the bond and the amount of invertase held.

Fischer and Kohtès (10) reported that highly purified invertase preparations contain 70 to 80% of mannan with 1,3 linkages. Repeated absorptions and elutions from aluminum hydroxide did not change the ratio of protein to mannan. When procedures were used that removed the mannan, the enzyme rapidly lost activity. Fischer and Kohtès believe the mannan and invertase are not a single compound but two that have great mutual affinity, and that the mannan confers stability upon an enzyme that is highly instable when pure. More recently, other workers have found the mannans associated with invertase preparations to have other linkages in addition to the 1,3 linkages reported in the preceding study. Although the invertase story is far from complete, it is advancing rapidly.

Wickerham and Dworschack (46) reported that a bisexual parent culture of *Saccharomyces kluyveri* produced only 26 units of extracellular invertase per milliliter of culture, but its sexually agglutinative ascosporic isolates produced much more, from 253 to 800 units. The intensity of the sexual agglutination reaction and the amounts of extracellular invertase increased with ploidy of the mating types.

F. Sexual Agglutination

Wickerham (39) discovered sexual agglutination in heterothallic species of yeasts in four genera. In strains possessing the reaction to a marked degree, cells of opposite sexes, whether grown in liquid or on solid media, flocculate immediately upon being mixed together. Brock (3) found in *H. wingei*, the first sexually agglutinative species studied, that one mating type carries on its surface a specific protein and that the other sex carries a specific carbohydrate which agglutinate together as do antigen and its specific antibody. The carbohydrate was not believed to be a mannan. Sneath and Lederberg (27) found that one of the sexes of *Escherichia coli* possesses a carbohydrate which is involved in mating, but they did not find a specific protein on the opposite sex.

Eddy (8) found certain pairs of brewing strains of *Saccharomyces* flocculate together toward the end of the fermentation to a greater extent than do the individual strains. He designates such flocculation as mutual agglutination. Eddy and Rudin (9) believe that the particular substances interacting during agglutination are proteins and mannans. Mutual agglutination is a very weak reaction compared with sexual agglutination in species such as *H. wingei* and *S. kluyveri*. Mutual agglutination (*unpublished data*) occurs between cells of bisexual strains; sexual agglutination occurs between mating types only. Eddy (8) emphasized that mutual agglutination resembles in its properties autoagglutination of individual strains. Perhaps mutual agglutination may also resemble sexual agglutination in mechanism, the difference being that all the elements involved occur in varying ratios on all cells in mutual agglutination and autoagglutination; but more highly reactive molecular species are sepa-

rated in sexual agglutination, so specific carbohydrates and specific proteins occur on opposite sexes. Presumably both types occur on the bisexuals of sexually agglutinative yeasts, as they do in the mutually agglutinative yeasts.

Brock (3) reported that pooled serum globulin inhibits the sexual agglutination reaction of *H. wingei*. Steinberg and Giles (29) found that all human sera cause some reduction of the reaction but that the sera of certain persons clearly cause a stronger inhibition than do the sera of others; their results were reported to be remarkably reproducible. About half of 353 sera contained the inhibitor. In another series of parents and offspring in 55 families, sexual agglutination of mating types 5 and 21 was not inhibited by the sera of offspring unless the serum of at least one parent also caused inhibition. Steinberg and Giles assume that the presence of the inhibiting factor is due to a dominant gene. Both mating types of *H. wingei*, brewer's yeast, or baker's yeast, used separately, absorb the inhibitor from active serum. Apparently the sexual agglutination reaction is useful in blood typing simply because of its convenience and not because of any inherent specificity due to the species of yeast. Studies on the serological nature of the inhibiting factor are being continued. Race (22) heralds the inhibition of sexual agglutination as another way in which bloods may be grouped.

We believe the principal function of sexual agglutination is to increase ploidy level within the species which possess it. This fact should be stressed since increase of ploidy is the most important process involved in the evolution of yeasts. In *H. wingei* and *Citeromyces matritensis*, sexual agglutination causes a very rapid conversion of opposite sexes of haploid mating types to diploid bisexuals. In *S. kluyveri*, sexual agglutination causes appropriate mixtures of haploid, diploid, and presumed polyploid mating types to mate and produce bisexuals ranging from diploid to tetraploid and probably even through octaploid. The rate of formation of polyploids is much faster in this species than in any species that is not sexually agglutinative. Sexual agglutination is a complex of mechanisms that are as yet little understood. They are intimately integrated with other highly complex sexual mechanisms. We are now engaged in a study the results of which should serve as an introduction to this fascinating field.

In closing, we reflect that the disciplines of chemistry, microbial genetics, and taxonomy may partake with mutual benefit in cooperative studies of the phylogeny of microorganisms. Our phylogenetic study of the genus *Hansenula*, based on characteristics commonly employed in the taxonomic study of yeasts, has made apparent evolutionary lines of species within the genus and between closely related genera. The discovery of new biochemicals and biological processes has strengthened these relationships and added to our knowledge of the types of compounds produced at different evolutionary levels.

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